



THE UNIVERSITY *of* EDINBURGH

## Edinburgh Research Explorer

### Hypothermia protects human neurons

**Citation for published version:**

Antonic, A, Dottori, M, Leung, J, Sidon, K, Batchelor, PE, Wilson, W, Macleod, MR & Howells, DW 2014, 'Hypothermia protects human neurons', *International Journal of Stroke*. <https://doi.org/10.1111/ijs.12224>

**Digital Object Identifier (DOI):**

[10.1111/ijs.12224](https://doi.org/10.1111/ijs.12224)

**Link:**

[Link to publication record in Edinburgh Research Explorer](#)

**Document Version:**

Publisher's PDF, also known as Version of record

**Published In:**

International Journal of Stroke

**Publisher Rights Statement:**

© 2014 The Authors. International Journal of Stroke published by John Wiley & Sons Ltd on behalf of World Stroke Organization.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

**General rights**

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

**Take down policy**

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact [openaccess@ed.ac.uk](mailto:openaccess@ed.ac.uk) providing details, and we will remove access to the work immediately and investigate your claim.



## Hypothermia protects human neurons

Ana Antonic<sup>1,2</sup>, Mirella Dottori<sup>3</sup>, Jessie Leung<sup>3</sup>, Kate Sidon<sup>1,2</sup>, Peter E. Batchelor<sup>2</sup>, William Wilson<sup>5</sup>, Malcolm R. Macleod<sup>4</sup>, and David W. Howells<sup>1,2\*</sup>

**Background and Aims** Hypothermia provides neuroprotection after cardiac arrest, hypoxic-ischemic encephalopathy, and in animal models of ischemic stroke. However, as drug development for stroke has been beset by translational failure, we sought additional evidence that hypothermia protects human neurons against ischemic injury.

**Methods** Human embryonic stem cells were cultured and differentiated to provide a source of neurons expressing  $\beta$  III tubulin, microtubule-associated protein 2, and the Neuronal Nuclei antigen. Oxygen deprivation, oxygen-glucose deprivation, and  $H_2O_2$ -induced oxidative stress were used to induce relevant injury.

**Results** Hypothermia to 33°C protected these human neurons against  $H_2O_2$ -induced oxidative stress reducing lactate dehydrogenase release and Terminal deoxynucleotidyl transferase dUTP nick end labeling-staining by 53% ( $P \leq 0.0001$ ; 95% confidence interval 34.8–71.04) and 42% ( $P \leq 0.0001$ ; 95% confidence interval 27.5–56.6), respectively, after 24 h in culture. Hypothermia provided similar protection against oxygen-glucose deprivation (42%,  $P \leq 0.001$ , 95% confidence interval 18.3–71.3 and 26%,  $P \leq 0.001$ ; 95% confidence interval 12.4–52.2, respectively) but provided no protection against oxygen deprivation alone. Protection (21%) persisted against  $H_2O_2$ -induced oxidative stress even when hypothermia was initiated six-hours after onset of injury ( $P \leq 0.05$ ; 95% confidence interval 0.57–43.1).

**Conclusion** We conclude that hypothermia protects stem cell-derived human neurons against insults relevant to stroke over a clinically relevant time frame. Protection against  $H_2O_2$ -induced injury and combined oxygen and glucose deprivation but not against oxygen deprivation alone suggests an interaction in which protection benefits from reduction in available glucose under some but not all circumstances.

Key words: brain, hypothermia, ischemic stroke, neuroprotection, stem cells, treatment

## Introduction

Obstruction of an artery supplying the brain initiates a cascade of events leading ultimately to necrotic and apoptotic cell death.

Correspondence: David W. Howells\*, Florey Institute of Neuroscience and Mental Health, 245 Burgundy Street, Heidelberg, Vic 3084, Australia. E-mail: david.howells@unimelb.edu.au

<sup>1</sup>Florey Institute of Neuroscience and Mental Health, Heidelberg, Vic, Australia

<sup>2</sup>Department of Medicine, University of Melbourne, Heidelberg, Vic, Australia

<sup>3</sup>Centre for Neuroscience Research, Department of Anatomy and Neuroscience, University of Melbourne, Melbourne, Vic, Australia

<sup>4</sup>Department of Clinical Neurosciences, Western General Hospital, University of Edinburgh, Edinburgh, UK

<sup>5</sup>CSIRO Mathematics, Informatics and Statistics, Riverside Life Sciences Precinct, North Ryde, NSW, Australia

Conflict of interest: None declared.

DOI: 10.1111/ijis.12224

With the most extreme perfusion deficits, survival is unlikely and cell death is rapid. However, in areas adjacent to the ischemic core (the ischemic penumbra), residual blood flow can preserve tissue vitality for a limited time until the obstruction resolves spontaneously or is removed by thrombolysis (1,2). After these acute events delayed neuronal death may progress for up to three-days (3). Both these phenomena (the penumbra during ischemia and delayed neural injury after ischemia) provide targets for treatment and in animal models of ischemic stroke over 500 'neuroprotective' treatment strategies reported improved outcome. However, none have proved robustly effective in randomized controlled clinical trials (4). Importantly with the exception of therapeutic hypothermia (5), clinical trials of neuroprotection for stroke have largely been abandoned (<http://www.clinicaltrials.gov>).

The reasons for this translational failure have been hotly debated. Contamination of the preclinical data set by falsely positive results influenced by bias and lack of statistical power offer some explanation (6–8). The molecular targets of therapy may be present in rodents but not in man (9), either because of differences in genetic background (10) or because evolution of the ischemic cascade might proceed at a different pace in different species (11–13). Additionally, laboratory and clinical thrombolysis experiments show similar, and unfortunately short, windows of opportunity (2,14), which are frequently unachievable in most trials of neuroprotection (15,16). Targeting single components of the ischemic cascade might also limit our chances of success (17,18), but combinatorial pharmacotherapy therapy brings its own challenges (19).

The preclinical data set for therapeutic hypothermia is large (3353 animals) and demonstrates substantial and consistent efficacy, with only modest effects of publication bias and failure to randomize or blind (20). Hypothermia influences multiple molecular targets (21,22) and provides benefit over a wide range of times to treatment in animals (20). Clinically, evidence of a relationship between body temperature and outcome after human stroke (23–25), together with the proven neuroprotective benefit of hypothermia in adults with global ischemia after cardiac arrest (26,27) and neonates with hypoxic-ischemic encephalopathy (28,29), suggest that the targets that provide benefit in animal models of stroke will also be present in human stroke. However, the absence of clear therapeutic benefit in traumatic brain injury (30), which accrues damage similarly to stroke (31), suggests caution should be taken. A Cochrane systematic review of stroke patients treated with physical cooling devices suggests there may be a trend toward improvement. However, in most cases, treatment was initiated (8–12 h) when penumbral tissue, the main putative target, may no longer be present. Overall, the analysis supports the view that we still have insufficient data

(32) with trials too small to allow firm conclusions to be made about the efficacy of hypothermia in stroke patients (33–35).

In this report, we show that hypothermia (33°C) protects human stem-cell derived neurons from oxidative stress induced by hydrogen peroxide and from oxygen-glucose deprivation but not hypoxia alone. Importantly, protection against oxidative stress is observed at later times (six-hours) than obtained under oxygen-glucose deprivation. The results were replicated in three independent series of experiments employing randomization and blinding of operator-dependent, outcome analyses.

## Methods

### Human embryonic stem cell (hESC) Culture

Experiments with these cells were carried out in accordance with the guidelines and regulations of the National Health and Medical Research Council and with the approval of the Austin Health Human Research Ethics Committee (Approval number H2008/03194) and University of Melbourne Human Research Ethics Committee (Approval number 0605017).

H9 (WA-09, WiCell, Madison, Wisconsin, USA), human embryonic stem cells (hESC), were cultured on mitomycin-C treated mouse embryonic fibroblasts (MEFs) in hESC medium consisting of high-glucose Dulbecco's modified Eagle's minimal essential medium (DMEM) without sodium pyruvate, supplemented with insulin/transferrin/selenium 1%,  $\beta$ -mercaptoethanol 0.1 mM, nonessential amino acids (NEAAs) 1%, glutamine 2 mM, penicillin 25 U/ml, streptomycin 25  $\mu$ g/ml (all from Invitrogen, Victoria, Australia) and fetal calf serum (FCS) 20% (HyClone, Australia) or on mitomycin-C treated human foreskin fibroblasts (HFF; ATCC, CRL-2097) in KSR media consisting of DMEM/nutrient mixture F-12, supplemented with  $\beta$ -mercaptoethanol 0.1 mM, NEAAs 1%, glutamine 2 mM, penicillin 25 U/ml, streptomycin 25  $\mu$ g/ml, and knockout serum replacement 20% (all from Invitrogen). All cells were cultured at 37°C in 5% CO<sub>2</sub>. Colonies were mechanically dissected every seven-days and transferred to freshly prepared MEFs or HFFs. Media was changed every second day.

### Neuronal differentiation and growth

Neuronal differentiation was achieved using the noggin induction method described for mouse neurospheres (36) as adapted by Dottori for human neurospheres (37). The colonies were maintained (37°C, 5% CO<sub>2</sub>) in hESC medium supplemented with 500 ng/ml of Noggin (6057-NG, R&D systems, Australia) for 14 days with the media and Noggin replaced every second day.

At this point, the colonies were again mechanically dissociated, but at this time the central (differentiated) part of the colony was also cut into smaller pieces using a 26-gauge needle. The pieces were transferred to individual wells in a low adherent 96-well plate containing Neural Basal Media (NBM) that contains Neurobasal A (10888-022, Invitrogen) supplemented with 2% B27 (17504-044, Invitrogen), 1% Insulin Transferrin Selenium-A (51500-056, Invitrogen), 1% N2 (17502-048, Invitrogen), 2 mM glutamine, 0.5% Penicillin-Streptomycin Solution (15070-063, Invitrogen), 20 ng/ml human recombinant epidermal growth

factor (EGF) (PHG0314, Invitrogen) and 20 ng/ml human recombinant basic fibroblast growth factor (bFGF) (13256-029, Invitrogen). Media was changed every two- to three-days to allow neurosphere formation over two-weeks.

In order to facilitate neuronal differentiation, neurospheres were again separated into smaller pieces under a dissection microscope with three to four pieces transferred to each well of a 96-well plate [precoated with poly-D-lysine (10  $\mu$ g/ml) and mouse laminin (5  $\mu$ g/ml)]. The cells were then grown for 11 days (with media changed every two-days) in NBM lacking growth factors prior to induction of injury and assessment of hypothermia.

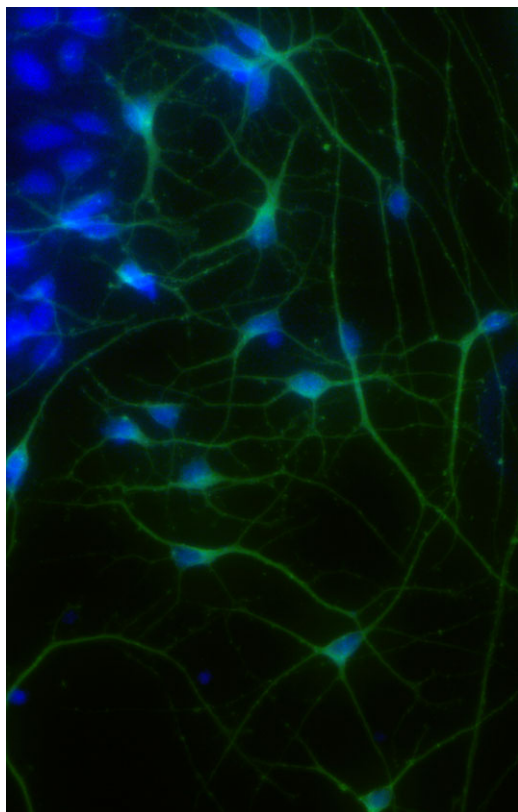
### Injury and hypothermia induction

On the day of experiments, the medium was changed to NBM+N2 containing a B27 preparation lacking the usual antioxidants (10889-038, Invitrogen) (NBM-AO) to eliminate their confounding effects.

Three different injury models were used to assess the effect of hypothermia on these cells. Oxygen deprivation was induced by placing the cells into a hypoxic chamber flushed with nitrogen for 20 min and maintained at the appropriate temperature (33°C or 37°C) for four-hours inside a standard 5% CO<sub>2</sub> incubator. Culture supernatant was removed after this four-hour period and stored at 4°C until analysed for lactate dehydrogenase activity (LDH). The media was replaced with fresh NBM-AO, and the cells were incubated for a further 20 h at the appropriate temperature (33°C or 37°C) before again measuring LDH. For combined oxygen and glucose deprivation (ODG), 25 mM 2-deoxy-D-glucose was added to NBM-AO medium and equilibrated for 30 min at room temperature before the initial media change as described above. The third injury model used was oxidative stress induced by adding 50  $\mu$ M of fresh H<sub>2</sub>O<sub>2</sub> (H1009, Sigma-Aldrich, Australia) to the NBM-AO for four-hours (33°C or 37°C, 5% CO<sub>2</sub>) when LDH was measured, and NBM-AO without H<sub>2</sub>O<sub>2</sub> was returned to the culture, which was maintained for a further 20 h before again measuring LDH. To examine whether the effects of injury continued beyond four-hours, the media containing the above stressors was removed and replaced with fresh growth factor negative NBM before resampling for LDH activity and assessment of apoptotic cell death by terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining at 24 h.

To evaluate the effects of hypothermia over time from the induction of injury, incubation at 33°C was started immediately, or one-, or three-, or six-hours after induction of injury and maintained until a total of 24 h in each of the models.

LDH, a marker of total cell death and TUNEL staining and a marker of DNA damage typical of apoptosis, were performed according to the kit manufacturer's instructions (1164479001 and 11684795910, respectively, Roche, Australia). For LDH assays, three control measurements were made in addition to the test sample measurements. The cell culture medium without cells was assayed to assess background activity. Uninjured cells were assayed to detect activity due to basal cell death, and maximum possible cell death (100%) was detected by measuring LDH activity of lysed cells.



**Fig. 1**  $\beta$  III tubulin positive human neurons after 11 days in culture with nuclei counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI).

### Statistical analysis

Each experiment was repeated three times. To avoid systematic effects on individual cultures because of the arrangement of wells in the tissue culture plates, control and injury cultures were distributed across the plates at random. Complete experiments contained within each tissue culture plate were then placed alternatively at either 37°C or 33°C. To minimize the potential impact of systematic bias, before counting of TUNEL positive cells, wells were imaged and the images recoded independently before quantitation. No additional blinding was performed for the machine-read LDH assay process. Within experiments, each comparison was performed at least in triplicate and the mean of these values taken forward into group comparisons. Two-way analysis of variance was performed, followed by post hoc Dunnett's multiple comparison test with significance set at  $P < 0.05$  using SPSS (Statistics 20, IBM, Armonk, NY, USA). All values are presented as mean  $\pm$  standard error of the mean.

### Results

The method of stem cell culture and neuronal differentiation employed in this study gives rise to neurons with a mature projecting phenotype (Fig. 1) that express the markers  $\beta$  III tubulin, microtubule-associated protein 2 (MAP2), and neural nuclei (NeuN).  $\beta$  III tubulin positive cells comprise  $70.4\% \pm 1.8$  of the cells present before injury (obtained by calculating the ratio of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) and  $\beta$  III

tubulin cells from 10 fields per well in three independent experiments). Dose response assays were performed to establish injury assay conditions (hypoxia, hypoxia plus glucose deprivation, and oxidative stress) that reproducibly induced 30–40% of maximal signal at four-hours to ensure that values lay within the working range of the assays used. In control cultures without injury, hypothermia had no effect on the basal level of cell death measured by LDH release at either 4 or 24 h (Figs 2–5).

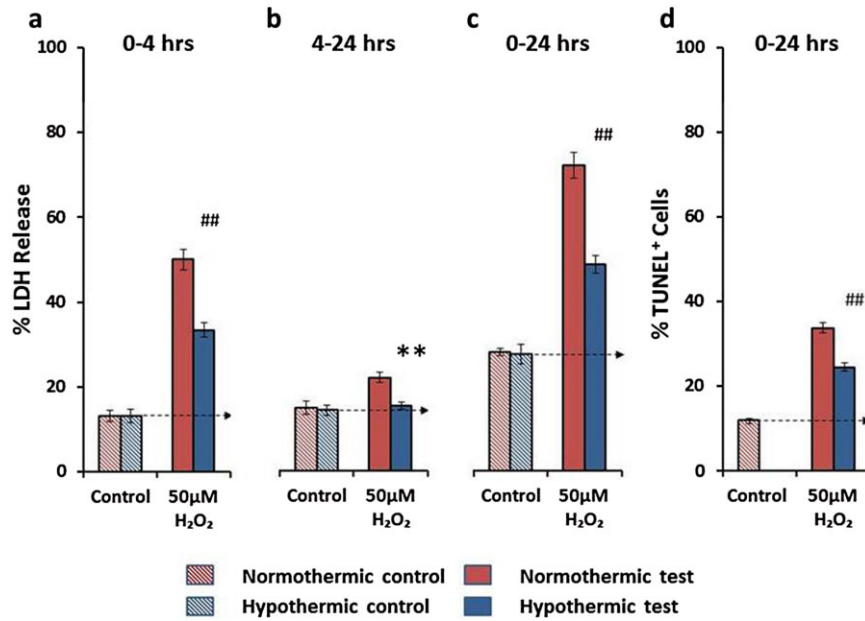
$H_2O_2$ -induced oxidative stress increased LDH-detected cell death 3.8-fold after four-hours. Removal of the  $H_2O_2$  by replacing the culture media at four-hours dramatically slowed but did not halt the  $H_2O_2$ -induced injury (Fig. 2a,b). Hypothermia to 33°C reduced  $H_2O_2$ -induced cell death at four-hours by 45% [ $P \leq 0.0001$ ; 95% confidence interval (CI) 27.5–62.6] (after correcting for basal injury in controls) (Fig. 2a). Hypothermia effectively abolished (92% reduction) ( $P \leq 0.001$ ; 95% CI 45.3–131.3) the delayed injury that continued to accrue between removal of  $H_2O_2$  at four-hours and completion of the experiment at 24 h (Fig. 2b). The net effect at 24 h was a reduction of LDH release of 53% ( $P \leq 0.0001$ ; 95% CI 34.8–71.04) (Fig. 2c).  $H_2O_2$ -induced oxidative stress increased TUNEL-detected apoptotic cell death 2.9-fold after 24 h, with 22% of cells present in culture killed by this mechanism upon introduction of  $H_2O_2$ . Hypothermia prevented 42% ( $P \leq 0.0001$ ; 95% CI 27.5–56.6) of this death (after correcting for basal injury in the control) (Fig. 2d).

Oxygen deprivation alone increased LDH-detected cell death approximately twofold after four-hours. Restoring the culture to a normal air/5%  $CO_2$  incubator and replacing the culture media at four-hours again slowed but did not completely halt the oxygen depletion induced injury (Fig. 3a,b). Hypothermia to 33°C had no discernible effect on oxygen depletion-induced cell death at either 4 or 24 h by either LDH or TUNEL assays (Fig. 3a–d).

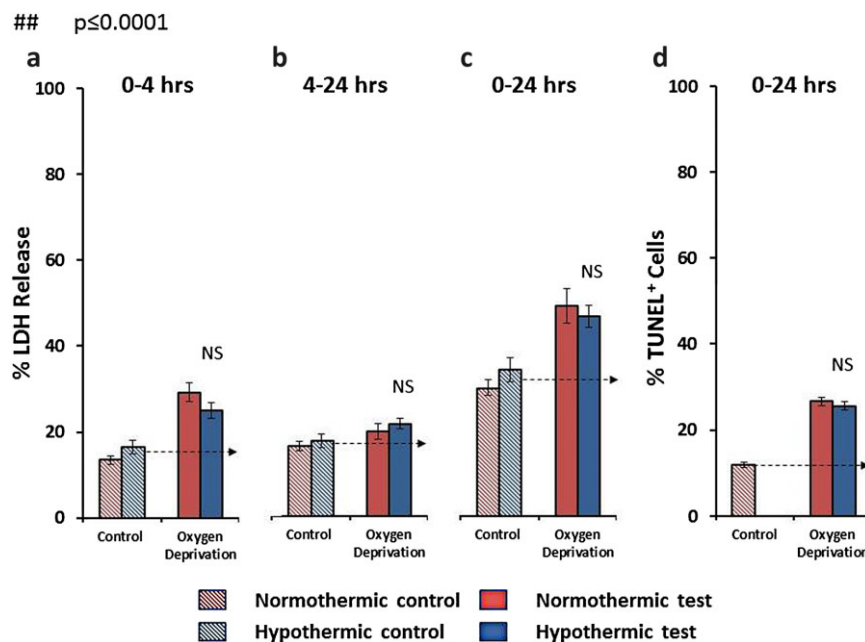
Combined OGD caused greater injury, increasing LDH-detected cell death 3.9-fold after four-hours. This death continued at a slower rate on restoration of normal culture conditions (Fig. 4a,b). Hypothermia to 33°C reduced OGD induced cell death at four-hours by 37% ( $P \leq 0.006$ ; 95% CI 6.8–44.8) (after correcting for basal injury in controls) (Fig. 4a). Hypothermia reduced the delayed injury that occurred between removal of OGD at four-hours and completion of the experiment at 24 h by 80% ( $P \leq 0.015$ ; 95% CI 13.8–146.8) (Fig. 4b). The net effect at 24 h was a reduction of LDH release of 42% ( $P \leq 0.001$ ; 95% CI 18.3–71.3) (Fig. 4c). TUNEL staining for DNA damage typical of apoptosis at 24 h suggested that 22% of cell death occurred by this mechanism and that hypothermia prevented 26% ( $P \leq 0.001$ ; 95% CI 12.4–52.2) of this death (after correcting for basal injury in the control) (Fig. 4d).

To confirm these observations, the  $H_2O_2$  and OGD experiments were repeated as before with hypothermia throughout the period of exposure to injury (from 0 h) and with initiation of hypothermia delayed for one-, three-, or six-hours, with outcome recorded as the total % of LDH release at 24 h. As before hypothermia had no impact on cell death in control cultures but when initiated at 0, one-, three-, or six-hours protected against  $H_2O_2$  toxicity by 52% ( $P \leq 0.0001$ ; 95% CI 29.8–73.5), 43% ( $P \leq 0.0001$ ; 95% CI 20.9–64.5), 34% ( $P \leq 0.001$ ; 95% CI 12.1–55.8), and 21% ( $P \leq 0.05$ ;





**Fig. 2** The effects of hypothermia on  $H_2O_2$ -induced cell death. Hypothermia reduces LDH detected cell death by 45% at four-hours (a) and effectively abolished (92% reduction) the delayed injury (b). The net effect at 24 h was reduction of 53% (c).  $H_2O_2$ -induced oxidative stress increased TUNEL-detected apoptotic cell death 2.9-fold after 24 h and hypothermia prevents 42% of this death (d). Statistical significance detected at  $*P \leq 0.05$ ,  $**P \leq 0.01$ ,  $\#P \leq 0.0005$ , and  $##P \leq 0.0001$ . Data presented as mean  $\pm$  SEM.



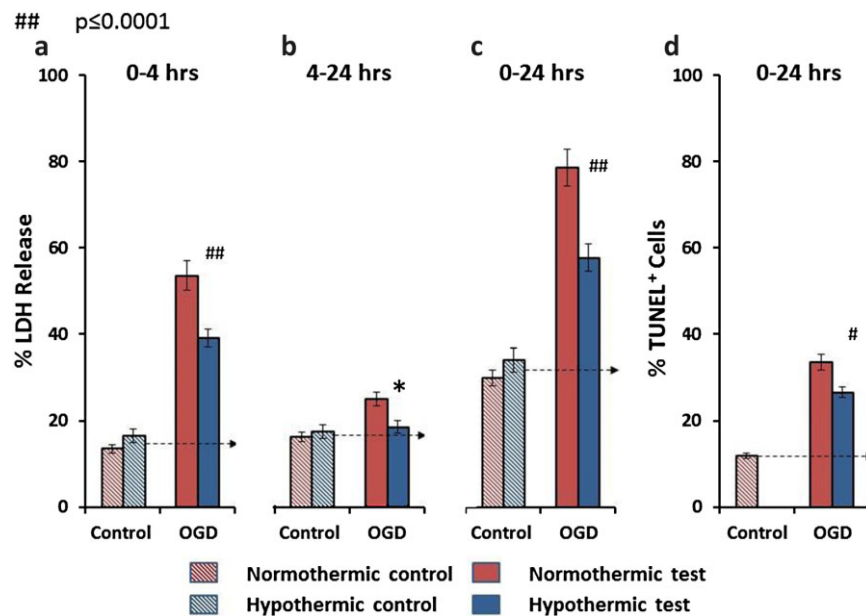
**Fig. 3** The effects of hypothermia on oxygen deprivation induced cell death. Hypothermia has no beneficial effect at four hours (a), 4–20 h (b), or in the combined 24 h LDH measurement of cell death (c) as well as on apoptotic cell death detected by TUNEL staining at 24 h (d). NS = not significant.

95% CI 0.57–43.1) (Fig. 5a), respectively, and against OGD-induced injury by 45% ( $P \leq 0.0004$ ; 95% CI 18.5–71.9), 30% ( $P \leq 0.023$ ; 95% CI 3.4–56.9), 27% ( $P \leq 0.041$ ; 95% CI 0.84–54.3), and 4% ( $P = 0.99$ ; 95% CI –23.02 to 30.4) (Fig. 5b), respectively.

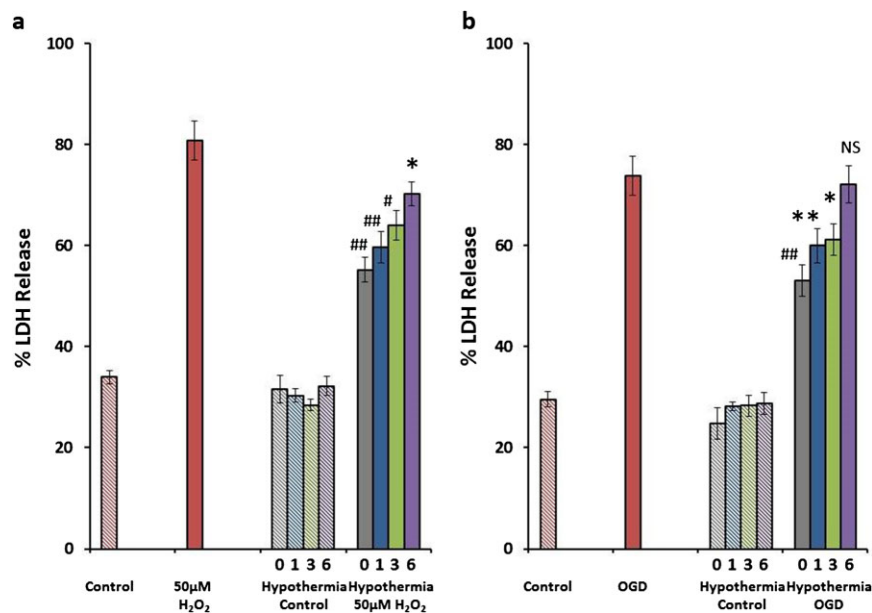
## Discussion

This study provides the first description of protection of embryonic stem cell-derived human neurons by hypothermia. This is

broadly consistent with data from ~20 publications (Table 1) that have examined the effects of hypothermia in tissues cultured from rats, mice, gerbils, guinea pigs, and three papers utilizing human teratoma lines (40–50,52,54–56). In this literature, glutamate release (40), calcium accumulation (38), and glucose utilization (45) are reduced while membrane potential (41,51) and cellular morphology are restored (49). These are consistent with reduced injury and cell death (39,44–46,48) measured by LDH release (40,45), propidium iodide uptake (46,48), 3-(4,5-



**Fig. 4** The effects of hypothermia on oxygen glucose deprivation (OGD)-induced cell death. Hypothermia reduced OGD-induced cell death at four hours by 37% (a). Hypothermia reduced the delayed injury that occurred between removal of OGD at four hours and completion of the experiment at 24 h by 80% (b). The net effect at 24 h was a reduction of LDH release of 42% (c). TUNEL staining for apoptosis at 24 h suggested that 22% of cell death occurred by this mechanism and that hypothermia prevented 26% of this death (d). \* $P \leq 0.05$ , # $P \leq 0.0005$ , and ## $P \leq 0.0001$ . Data presented as mean  $\pm$  SEM.



**Fig. 5** Time-dependent effects of hypothermia on H<sub>2</sub>O<sub>2</sub> and OGD-induced cell death. Hypothermia reduces LDH-detected cell death induced with H<sub>2</sub>O<sub>2</sub> by 52%, 43%, 34%, and 21% (a) and OGD-induced injury by 45%, 30%, 27%, and 4% when started at 0, one-, three-, and six-hours (b), respectively. \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , # $P \leq 0.0005$ , and ## $P \leq 0.0001$ . Data presented as mean  $\pm$  SEM.

dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining (54), caspase activity (44), and other immunohistochemical techniques (42,50,52,53,57).

Hypothermia significantly protected embryonic stem cell-derived human neurons against oxidative stress and from hypoxia when glucose concentration was reduced but was ineffective against hypoxia alone. Neuroprotective effects were seen with delays in initiation of hypothermia of up to six-hours, with the magnitude of benefit progressively decreasing as the time to

hypothermia initiation increased. In our preparations protection against apoptosis was also detected, consistent with data from mouse neuronal cultures (44) and focal cerebral ischemia experiments in animals (58) showing that mild hypothermia attenuates DNA damage typical of apoptotic neuronal cell death. However, the difference between degree of injury detected at 24 h by LDH and TUNEL assays suggests cell death is also occurring in the cultures by nonapoptotic mechanisms such as necrosis.

**Table 1** Summary of hypothermia use to protect cells *in vitro*

Year	Hypothermia temperature	Type of culture	Injury	Duration of injury	Outcome measure	Outcome	Reference
1991	35°C, 33°C, and 31°C	Gerbil hippocampal slices	OGD	Unknown	Intracellular Ca <sup>2+</sup> accumulation	Ca <sup>2+</sup> accumulation was delayed following hypothermia treatment	(38)
1992	32°C	Rat cerebellar granule cells and GABAergic neurons	OGD	3–6 h	LDH	Neuroprotective, reduces LDH caused cell death	(39)
1998	33°C	Guinea pig E40, E60, and adult slices	OGD	2 h and 12 h	HPLC	Reduced glutamate release was only observed in the immature cultures	(40)
1998	35°C, 33°C, and 27°C	Rat hippocampal slices	OGD	Unknown	Extracellular membrane potential recordings	27°C and 33°C the membrane potential was restored to the control conditions	(41)
2000	34°C	Rat hippocampal slices	OGD	10–20 min	Immunohistochemistry	Hypothermia reduced CA1 cell loss	(42)
2001	31°C	Guinea pig hippocampal slices	OGD	20, 30, or 40 min	Recovery of energy metabolism	When hypothermia added at 0 or 2 h postinjury induction it reduced energy metabolic rate	(43)
2002	33°C	Rat cortical cultures	OGD	Unknown	Caspase 3–8 and –9 activity	Hypothermia neuronal apoptotic cell death	(44)
2003	30°C and 33°C	Rat retina	OGD	1–2 h	LDH	Hypothermia reduced glucose utilization and lactate production	(45)
2004	31°C, 33°C, and 35°C	Rat hippocampal slices	OGD	60 min	Propidium iodide (PI)	Reduced PI detected cell death only at 31°C and 33°C	(46)
2004	33°C preconditioning	Rat cerebellar slices	OGD	20 min	Immunohistochemistry	Hypothermic preconditioning induces acute neuroprotection	(47)
2005	35°C	Rat hippocampal slices	OGD	1 h	Propidium iodide	Protects all regions of hippocampus from cell death	(48)
2005	27°C or 33°C	Dissociated mouse hippocampal neurons	OGD	10–25 min	Spine shape and motility assessed by microscopy	Decrease in temperature reduced the spine motility, and the length of spines was reduced	(49)
2005	35°C and 31°C	Rat hippocampal slices	OGD	40 min	Immunohistochemistry	Decrease in temperature – increased protection in all the hippocampal regions	(50)
2006	34°C pre treatment	Rat hippocampal slices	OGD	20 min	Electrophysiology	Temperature lowering reduced OGD-induced cell death	(51)
2006	34°C, 30°C, or 22°C	Rat hippocampal neurons	OGD	Unknown	Expression of Bcl-2, fluorescence magnitude of intracellular Ca <sup>2+</sup>	Expression of Bcl-2 was increased with decrease in temperature	(52)
2006	33°C preconditioning for 20 min, 1 h before OGD	Rat cerebellar cells	OGD	20 min	Immunohistochemistry	Hypothermic preconditioning increased survival of Purkinje neurons in rat cerebellar slices after OGD	(53)
2009	33°C	Human NT2-N neurons	OGD	20 min	MTT	Posthypoxic hypothermia is protective in human NT2-N neurons	(54)
2010	33°C and 30°C	Rat cortical neurons	Hypoxia	24 h	Cell viability	Increased cell survival following hypothermia	(55)

There are a number of studies using NT2-N neurons in similar experiments with conflicting data evident. Although one study reports essentially the same result as here (54), another reports that hypothermia protects against the effects of oxygen restriction alone (59). The reasons for these differences are unclear but suggest differential expression of the metabolic or signaling machinery involved in ischemic injury. NT2-N cells are derived from the human NTera2 embryonal carcinoma stem cell line (60) and are known to differ substantially in their broad pattern of gene expression when compared with different hESC lines (61) and therefore may not be the most appropriate models to study human disease (62).

Intriguingly, hypothermia had no effect on the basal level of cell death in the uninjured controls. The contrasting response to different injury models, lack of effect on culture dependent cell death, and different behaviors to experiments reported in other cell lines suggests interaction specifically in the processes of ischemic injury rather than cell death itself and requires further study. These differences also do not appear to be consistent with hypothermia-inducing protection simply by slowing overall metabolic activity (63) but could still be consistent with decreasing the cerebral metabolic rates of glucose and oxygen consumption (64) and slowing adenosine triphosphate (ATP) breakdown (65).

It is not clear how the time frame of protection of human neurons *in vitro* will be predictive of time frame in stroke. However, detection of protection against oxidative stress even after cessation of injury offers hope for the clinical setting. This may be particularly relevant if reperfusion injury (66–68) acts to lessen the potential benefit afforded by thrombolysis or thrombectomy and suggests this may be an important target for clinical trials of hypothermia. The data are certainly consistent with the recent observation that mild hypothermia reduces the deleterious side effects of tissue plasminogen activator treatment after thromboembolic stroke in rats (69). Different time frames of benefit via different mechanisms for a single therapeutic approach suggest we still have much to learn about the details of the ischemic cascade and the therapeutic opportunities it might offer. The window of opportunity detected here in human neurons *in vitro* is certainly consistent with a wide window in rodent models of stroke (20) and with the planned six-hour window for EuroHYP-1 (70).

As in most experiments, our data and its interpretation have limits. The current experiments have been performed in cells that exhibit a branching phenotype expressing  $\beta$  III tubulin. However, these cells have spent only 11 days in culture and thus might be better viewed as immature. It is not yet clear whether these cells can stand in for a 60-year-old neuron from a typical stroke victim. However, it seems reasonable to believe that we can devise strategies to rapidly and repeatedly stress them much as a car manufacturer would stress test a car. Nevertheless, we might see different effects when cultures have matured neurochemically. Moreover, clinically relevant protection might be dependent on effects on neurotransmission itself (71) or regulation of edema (72). Furthermore, beneficial effects might be restricted to specific neurochemical profiles present at high abundance in our culture but

not in areas of the brain affected by most common forms of human stroke. Another limitation of the current experiments was ethical approval to study only one hESC cell line. It will be important to confirm these observations in other hESC lines, and when the technology is more mature (73,74) to examine whether the same effects are seen in induced pluripotent stem cells (iPSCs) from patients with stroke or relevant defects of oxidative metabolism such as seen in inborn errors of mitochondrial function.

## Conclusion

This study provides the first description of protection of embryonic stem cell derived human neurons by hypothermia. If hypothermia does have a predilection for ischemic processes, this offers hope for use of hypothermia to treat ischemic stroke and spinal cord injuries where cord compression results in secondary ischemia (75). This study also provides proof of principle that human neurons derived from hESCs can be used to screen new drugs for therapeutic effect. For screening large chemical libraries, this will be more specific than screening in cell lines from other species, and more cost-effective than initial screening in animal models of stroke.

## Acknowledgements

We thank Clare Parish for her contributions toward this study.

The Florey Institute of Neuroscience and Mental Health acknowledges the strong support from the NHMRC and the Victorian Government and in particular the funding from the Operational Infrastructure Support Grant.

## References

- 1 Dirnagl U, Iadecola C, Moskowitz MA. Pathobiology of ischaemic stroke: an integrated view. *Trends Neurosci* 1999; **22**:391–7.
- 2 Lees KR, Bluhmki E, von Kummer R *et al.* Time to treatment with intravenous alteplase and outcome in stroke: an updated pooled analysis of ECASS, ATLANTIS, NINDS, and EPITHET trials. *Lancet [Meta-Analysis]* 2010; **375**:1695–703.
- 3 Pulsinelli WA, Brierley JB, Plum F. Temporal profile of neuronal damage in a model of transient forebrain ischemia. *Ann Neurol* 1982; **11**:491–8.
- 4 O'Collins VE, Macleod MR, Donnan GA, Horky LL, van der Worp BH, Howells DW. 1,026 experimental treatments in acute stroke. *Ann Neurol* 2006; **59**:467–77.
- 5 Kollmar R, Gebhardt B, Schwab S. [EuroHYP-1 trial : EU-funded therapy study on the effectiveness of mild therapeutic hypothermia for acute ischemic stroke.]. *Nervenarzt* 2012; **83**:1252–9.
- 6 Sena ES, van der Worp HB, Bath PM, Howells DW, Macleod MR. Publication bias in reports of animal stroke studies leads to major overstatement of efficacy. *PLoS Biol* 2010; **8**:e1000344.
- 7 Macleod MR, van der Worp HB, Sena ES, Howells DW, Dirnagl U, Donnan GA. Evidence for the efficacy of NXY-059 in experimental focal cerebral ischaemia is confounded by study quality. *Stroke* 2008; **39**:2824–9.
- 8 Macleod MR, O'Collins T, Horky LL, Howells DW, Donnan GA. Systematic review and metaanalysis of the efficacy of FK506 in experimental stroke. *J Cereb Blood Flow Metab* 2005; **25**:713–21.
- 9 Antonic A, Howells DW. Human *in vitro* models of ischaemic stroke: a test bed for translation. *Translational Stroke Research* 2012; **3**:306–9.



- 10 Zhao S, Shetty J, Hou L *et al*. Human, mouse, and rat genome large-scale rearrangements: stability versus speciation. *Genome Res* 2004; **14**(10A):1851–60.
- 11 Davalos A, Castillo J, Serena J, Noya M. Duration of glutamate release after acute ischemic stroke. *Stroke* [Research Support, Non-U.S. Gov't] 1997; **28**:708–10.
- 12 Takagi K, Ginsberg MD, Globus MY *et al*. Changes in amino acid neurotransmitters and cerebral blood flow in the ischemic penumbral region following middle cerebral artery occlusion in the rat: correlation with histopathology. *J Cereb Blood Flow Metab* 1993; **13**:575–85.
- 13 Nilupul Perera M, Ma HK, Arakawa S *et al*. Inflammation following stroke. *J Clin Neurosci* 2006; **13**:1–8.
- 14 Sena ES, Briscoe CL, Howells DW, Donnan GA, Sandercock PA, Macleod MR. Factors affecting the apparent efficacy and safety of tissue plasminogen activator in thrombotic occlusion models of stroke: systematic review and meta-analysis. *J Cereb Blood Flow Metab* 2010; **30**:1905–13.
- 15 Kidwell CS, Liebeskind DS, Starkman S, Saver JL. Trends in acute ischemic stroke trials through the 20th century. *Stroke* 2001; **32**:1349–59.
- 16 Ferguson KN, Kidwell CS, Starkman S, Saver JL. Hyperacute treatment initiation in neuroprotective agent stroke trials. *J Stroke Cerebrovasc Dis* 2004; **13**:109–12.
- 17 Recommendations for standards regarding preclinical neuroprotective and restorative drug development. *Stroke* 1999; **30**:2752–8.
- 18 Gladstone DJ, Black SE, Hakim AM, Heart, Stroke Foundation of Ontario Centre of Excellence in Stroke R. Toward wisdom from failure: lessons from neuroprotective stroke trials and new therapeutic directions. *Stroke* 2002; **33**:2123–36.
- 19 O'Collins VE, Macleod MR, Donnan GA, Howells DW. Evaluation of combination therapy in animal models of cerebral ischemia. *J Cereb Blood Flow Metab* [Review] 2012; **32**:585–97.
- 20 van der Worp HB, Sena ES, Donnan GA, Howells DW, Macleod MR. Hypothermia in animal models of acute ischaemic stroke: a systematic review and meta-analysis. *Brain* 2007; **130**:3063–74.
- 21 Zhao H, Steinberg GK, Sapolsky RM. General versus specific actions of mild-moderate hypothermia in attenuating cerebral ischemic damage. *J Cereb Blood Flow Metab* 2007; **27**:1879–94.
- 22 Yenari M, Kitagawa K, Lyden P, Perez-Pinzon M. Metabolic down-regulation: a key to successful neuroprotection? *Stroke* 2008; **39**:2910–7.
- 23 Reith J, Jorgensen HS, Pedersen PM *et al*. Body temperature in acute stroke: relation to stroke severity, infarct size, mortality, and outcome. *Lancet* 1996; **347**:422–5.
- 24 Castillo J, Davalos A, Marrugat J, Noya M. Timing for fever-related brain damage in acute ischemic stroke. *Stroke* [Research Support, Non-U.S. Gov't] 1998; **29**:2455–60.
- 25 den Hertog HM, van der Worp HB, van Gemert HM *et al*. The Paracetamol (Acetaminophen) In Stroke (PAIS) trial: a multicentre, randomised, placebo-controlled, phase III trial. *Lancet Neurol* 2009; **8**:434–40.
- 26 Bernard SA, Gray TW, Buist MD *et al*. Treatment of comatose survivors of out-of-hospital cardiac arrest with induced hypothermia. *N Engl J Med* 2002; **346**:557–63.
- 27 Arrich J, Holzer M, Herkner H, Mullner M. Hypothermia for neuroprotection in adults after cardiopulmonary resuscitation. *Cochrane Database Syst Rev* 2009; (4):CD004128.
- 28 Shankaran S, Laptook AR, Ehrenkranz RA *et al*. Whole-body hypothermia for neonates with hypoxic-ischemic encephalopathy. *N Engl J Med* 2005; **353**:1574–84.
- 29 Jacobs S, Hunt R, Tarnow-Mordi W, Inder T, Davis P. Cooling for newborns with hypoxic ischaemic encephalopathy. *Cochrane Database Syst Rev* 2007; (4):CD003311.
- 30 Sydenham E, Roberts I, Alderson P. Hypothermia for traumatic head injury. *Cochrane Database Syst Rev* 2009; (2):CD001048.
- 31 Leker RR, Shohami E. Cerebral ischemia and trauma-different etiologies yet similar mechanisms: neuroprotective opportunities. *Brain Res Brain Res Rev* 2002; **39**:55–73.
- 32 Den Hertog HM, van der Worp HB, Tseng MC, Dippel DW. Cooling therapy for acute stroke. *Cochrane Database Syst Rev* 2009; (1):CD001247.
- 33 De Georgia MA, Krieger DW, Abou-Chebl A *et al*. Cooling for Acute Ischemic Brain Damage (COOL AID): a feasibility trial of endovascular cooling. *Neurology* 2004; **63**:312–7.
- 34 Guluma KZ, Hemmen TM, Olsen SE, Rapp KS, Lyden PD. A trial of therapeutic hypothermia via endovascular approach in awake patients with acute ischemic stroke: methodology. *Acad Emerg Med* [Multicenter Study Randomized Controlled Trial] 2006; **13**:820–7.
- 35 Hemmen TM, Raman R, Guluma KZ *et al*. Intravenous thrombolysis plus hypothermia for acute treatment of ischemic stroke (ICTuS-L): final results. *Stroke* 2010; **41**:2265–70.
- 36 Reynolds BA, Tetzlaff W, Weiss S. A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. *J Neurosci* 1992; **12**:4565–74.
- 37 Dottori M, Pera MF. Neuronal differentiation of human embryonic stem cells. *Methods Mol Biol* 2007; **438**:19–30.
- 38 Mitani A, Kataoka K. Critical levels of extracellular glutamate mediating gerbil hippocampal delayed neuronal death during hypothermia: brain microdialysis study. *Neuroscience* 1991; **42**:661–70.
- 39 Yue TL, Gu JL, Lysko PG, Cheng HY, Barone FC, Feuerstein G. Neuroprotective effects of phenyl-t-butyl-nitron in gerbil global brain ischemia and in cultured rat cerebellar neurons. *Brain Res* 1992; **574**:193–7.
- 40 Berger R, Jensen A, Hossmann KA, Paschen W. Effect of mild hypothermia during and after transient in vitro ischemia on metabolic disturbances in hippocampal slices at different stages of development. *Brain Res Dev Brain Res* 1998; **105**:67–77.
- 41 Onitsuka M, Mihara S, Inokuchi H, Shigemori M, Higashi H. Mild hypothermia protects rat hippocampal CA1 neurons from irreversible membrane dysfunction induced by experimental ischemia. *Neurosci Res* 1998; **30**:1–6.
- 42 Popovic R, Liniger R, Bickler PE. Anesthetics and mild hypothermia similarly prevent hippocampal neuron death in an in vitro model of cerebral ischemia. *Anesthesiology* 2000; **92**:1343–9.
- 43 Garnier Y, Pfeiffer D, Jensen A, Berger R. Effects of mild hypothermia on metabolic disturbances in fetal hippocampal slices after oxygen/glucose deprivation depend on depth and time delay of cooling. *J Soc Gynecol Investig* 2001; **8**:198–205.
- 44 Xu L, Yenari MA, Steinberg GK, Giffard RG. Mild hypothermia reduces apoptosis of mouse neurons in vitro early in the cascade. *J Cereb Blood Flow Metab* 2002; **22**:21–8.
- 45 Quinones-Hinojosa A, Malek JY, Ames A, 3rd, Ogilvy CS, Maynard KI. Metabolic effects of hypothermia and its neuroprotective effects on the recovery of metabolic and electrophysiological function in the ischemic retina in vitro. *Neurosurgery* 2003; **52**:1178–86; discussion 86–7.
- 46 McManus T, Sadgrove M, Pringle AK, Chad JE, Sundstrom LE. Intracerebral hypothermia reduces free radical production and protects against ischaemic insults in cultured hippocampal slices. *J Neurochem* 2004; **91**:327–36.
- 47 Yuan HB, Huang Y, Zheng S, Zuo Z. Hypothermic preconditioning increases survival of purkinje neurons in rat cerebellar slices after an in vitro simulated ischemia. *Anesthesiology* 2004; **100**:331–7.
- 48 Feiner JR, Bickler PE, Estrada S, Donohoe PH, Fahlman CS, Schuyler JA. Mild hypothermia, but not propofol, is neuroprotective in organotypic hippocampal cultures. *Anesth Analg* 2005; **100**:215–25.
- 49 Gisselsson LL, Matus A, Wieloch T. Actin redistribution underlies the sparing effect of mild hypothermia on dendritic spine morphology after in vitro ischemia. *J Cereb Blood Flow Metab* 2005; **25**:1346–55.
- 50 Rytter A, Cardoso CM, Johansson P *et al*. The temperature dependence and involvement of mitochondria permeability transition and caspase activation in damage to organotypic hippocampal slices following in vitro ischemia. *J Neurochem* 2005; **95**:1108–17.

- 51 Lipski J, Park TI, Li D *et al.* Involvement of TRP-like channels in the acute ischemic response of hippocampal CA1 neurons in brain slices. *Brain Res* 2006; **1077**:187–99.
- 52 Liu RG, Wang WJ, Song N, Chen YQ, Li LH. Diazoxide preconditioning plus subsequent hypothermia increased resistance of rat cultured hippocampal neurons against hypoxia-reoxygenation injury. *Chin Med J (Engl)* 2006; **119**:887–93.
- 53 Yuan HB, Huang Y, Zheng S, Zuo Z. Hypothermic preconditioning reduces Purkinje cell death possibly by preventing the over-expression of inducible nitric oxide synthase in rat cerebellar slices after an in vitro simulated ischemia. *Neuroscience* 2006; **142**:381–9.
- 54 Dalen ML, Froyland E, Saugstad OD, Mollnes TE, Rootwelt T. Post-hypoxic hypothermia is protective in human NT2-N neurons regardless of oxygen concentration during reoxygenation. *Brain Res* 2009; **1259**:80–9.
- 55 Hua Y, Hisano K, Morimoto Y. Effect of mild and moderate hypothermia on hypoxic injury in nearly pure neuronal culture. *J Anesth* 2010; **26**:67–76.
- 56 Mitani A, Kadoya F, Kataoka K. Temperature dependence of hypoxia-induced calcium accumulation in gerbil hippocampal slices. *Brain Res* 1991; **562**:159–63.
- 57 Yuan HB, Huang YM, Zheng SQ, Zuo ZY. Hypothermic preconditioning increases survival of Purkinje neurons in rat cerebellar slices after an in vitro simulated ischemia. *Anesthesiology* 2004; **100**:331–7.
- 58 Eberspacher E, Werner C, Engelhard K *et al.* Long-term effects of hypothermia on neuronal cell death and the concentration of apoptotic proteins after incomplete cerebral ischemia and reperfusion in rats. *Acta Anaesthesiol Scand* 2005; **49**:477–87.
- 59 Rootwelt T, Dunn M, Yudkoff M, Itoh T, Almaas R, Pleasure D. Hypoxic cell death in human NT2-N neurons: involvement of NMDA and non-NMDA glutamate receptors. *J Neurochem* 1998; **71**:1544–53.
- 60 Pleasure SJ, Page C, Lee VM. Pure, postmitotic, polarized human neurons derived from NTera 2 cells provide a system for expressing exogenous proteins in terminally differentiated neurons. *J Neurosci* 1992; **12**:1802–15.
- 61 Schwartz CM, Spivak CE, Baker SC *et al.* NTera2: a model system to study dopaminergic differentiation of human embryonic stem cells. *Stem Cells and Development*. 2005; **14**:517–34.
- 62 Woodruff TM, Thundiyil J, Tang SC, Sobey CG, Taylor SM, Arumugam TV. Pathophysiology, treatment, and animal and cellular models of human ischemic stroke. *Mol Neurodegener* 2011; **6**:11.
- 63 Axelrod YK, Diringer MN. Temperature management in acute neurologic disorders. *Neurol Clin [Review]* 2008; **26**:585–603, xi.
- 64 Hagerdal M, Harp J, Nilsson L, Siesjö BK. The effect of induced hypothermia upon oxygen consumption in the rat brain. *J Neurochem* 1975; **24**:311–6.
- 65 Erecinska M, Thoresen M, Silver IA. Effects of hypothermia on energy metabolism in mammalian central nervous system. *J Cereb Blood Flow Metab* 2003; **23**:513–30.
- 66 Mowlavi A, Neumeister MW, Wilhelmi BJ, Song Y-H, Suchy H, Russell RC. Local hypothermia during early reperfusion protects skeletal muscle from ischemia-reperfusion injury. *Plastic Reconstr Surg* 2003; **111**:242–50.
- 67 Strbian D, Karjalainen-Lindsberg M-L, Kovanen PT, Tatlisumak T, Lindsberg PJ. Mast cell stabilization reduces hemorrhage formation and mortality after administration of thrombolytics in experimental ischemic stroke. *Circulation* 2007; **116**:411–8.
- 68 Aronowski J, Strong R, Grotta JC. Reperfusion injury: demonstration of brain damage produced by reperfusion after transient focal ischemia in rats. *J Cerebral Blood Flow Metab* 1997; **17**:1048–56.
- 69 Kallmunzer B, Schwab S, Kollmar R. Mild hypothermia of 34 degrees C reduces side effects of rt-PA treatment after thromboembolic stroke in rats. *Exp Transl Stroke Med* 2012; **4**:3.
- 70 van der Worp HB, Macleod MR, Kollmar R. Therapeutic hypothermia for acute ischemic stroke: ready to start large randomized trials[quest]. *J Cereb Blood Flow Metab* 2010; **30**:1079–93.
- 71 Yang XF, Ouyang Y, Kennedy BR, Rothman SM. Cooling blocks rat hippocampal neurotransmission by a presynaptic mechanism: observations using 2-photon microscopy. *J Physiol* 2005; **567**(Pt 1):215–24.
- 72 Schreckinger M, Marion DW. Contemporary management of traumatic intracranial hypertension: is there a role for therapeutic hypothermia? *Neurocrit Care [Review]* 2009; **11**:427–36.
- 73 Urbach A, Bar-Nur O, Daley GQ, Benvenisty N. Differential modeling of Fragile X syndrome by human embryonic stem cells and induced-pluripotent stem cells. *Cell Stem Cell* 2010; **6**:407.
- 74 Feng Q, Lu SJ, Klimanskaya I *et al.* Hemangioblastic derivatives from human induced pluripotent stem cells exhibit limited expansion and early senescence. *Stem Cells* 2010; **28**:704–12.
- 75 Tator CH, Fehlings MG. Review of the secondary injury theory of acute spinal cord trauma with emphasis on vascular mechanisms. *J Neurosurg* 1991; **75**:15–26.